REMARKS

Claims 1-5 are pending in this application. While box 4a of the Office Action Summary indicates that claims 3 and 4 have been withdrawn from consideration, Applicants note that claims 3-5 are indicated as being withdrawn from consideration in the Detailed Action. Claims 3-5 have been canceled. Claims 1 and 2 have been amended. No new matter has been added. Applicants are respectfully requesting reconsideration in light of the following remarks.

I. Election/Restriction Requirement Under 35 U.S.C. §121

The restriction requirement placing the claims into Groups I-IV has been deemed proper and made final. Thus, claims 3-5 have been withdrawn from further consideration. Accordingly, Applicants are canceling claims 3-5 without prejudice, reserving the right to file continuing applications for the canceled subject matter.

II. Priority

Priority to PCT/US99/26610, filed November 10, 1999 and provisional application 60/110,266, filed November 30, 1998 has been acknowledged; however, it is suggested that in order to receive the benefit of the earlier filing date under 35 U.S.C. 119, the instant specification must contain specific reference to the prior applications in the first sentence of the specification. Accordingly, Applicants have amended the specification to incorporate reference to priority applications PCT/US99/26610 and provisional application 60/110,26698.

III. Objection to the Specification

The specification has been objected to for containing an embedded hyperlink and/or other form of browser-executable code. Accordingly, Applicants have amended the paragraph at page 8 removing the browser-executable code.

IV. Rejection of Claims Under 35 U.S.C. §112

Claims 1-2 have been rejected under 35 U.S.C. 112, first paragraph, for failing to meet the written description requirement. The Examiner suggests that the specification teaches an insertion of a G at position -1607; however, does not explicitly state the position of the insertion in SEQ ID NO:3 that results in the Ets transcription factor binding site single nucleotide polymorphism set forth in the instant claims. It is suggested that amending the claim to specifically be directed to the -1607 polymorphism and a SEQ ID NO: would overcome this rejection.

MPEP 2163.02 indicates that an objective standard for determining compliance with the written description requirement is, "does the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed." In re Gosteli, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989). Whenever the issue arises, the fundamental factual inquiry is whether a claim defines an invention that is clearly conveyed to those skilled in the art at the time the application was filed. The subject matter of the claim need not be described literally (*i.e.*, using the same terms or in haec verba) in order for the disclosure to satisfy the description requirement.

The instant specification teaches at the paragraph bridging pages 6-7 and at page 7, lines 2-3, that an MMP-1 promoter fragment of the sequence 5'-AAA TAA TTA GAA AG ATA TGA CTT ATC TCA AAT CAA-3' (SEQ ID NO: 6) encompasses the location of the -1607 Ets transcription factor binding site single nucleotide polymorphism (SNP). As would be understood by the skilled artisan based upon the teachings provided at the paragraph bridging pages 2-3, an Ets transcription factor binding site SNP results in the conversion of 5'-AAGAT-3' to 5'-AAGGAT-3'. Therefore, to reflect these teachings in the instant claims, Applicants have amended claims 1 and 2 to recite that the 5'-AAGAT-3' to 5'-AAGGAT-3' Ets transcription factor binding site SNP is detected in the matrix metalloproteinase-1 promoter sequence comprising SEQ ID NO:6. Based upon these amendments, one of skill in the art would readily recognize that Applicants have invented what is claimed. It is therefore respectfully requested that this rejection be reconsidered and withdrawn.

Claims 1-2 have further been rejected under 35 U.S.C. 112, first paragraph, for failing to comply with the enablement requirement. The Examiner suggests that the claims are broadly drawn to detecting any metalloproteinase-1 related disease by detecting "the Ets transcription factor binding site polymorphism" which has not been specifically set forth in the claims in any patient. It is further suggested that the art supports the unpredictability of the broadly drawn claims as Lee et al. ((2003) *Scand. J. Rheumatol.* 32:235-39) and Constantin et al. ((2002) *J. Rheumatol.* 29:15-20) teach that the genotype distribution of the MMP-1 promoter does not differ between rheumatoid arthritis patients and control subjects; Jurajda et

al. ((2001) *Gynecol. Obstet Invest.* 52: 124-27) teach that there is a lack of association between a single nucleotide polymorphism in the promoter of the MMP-1 gene in Czech women with pregnancy-induced hypertension; Zhang et al. ((2001) *Stroke* 32:2198-2202) disclose that no significant difference is detected between the patient and control groups in relation of subarachnoid hemorrhage; Johnson et al. ((2001) *Genes Immun.* 2:273) teach the lack of association between a functionally relevant single nucleotide polymorphism with MMP-1 and systemic sclerosis; Louka et al. ((2001) *Scand. J. Gastroenterol.* 8:931-35) teach that celiac disease is not associated with a functional polymorphism in MMP-1 gene promoter; and Matsumura et al. ((2004) *J. Cancer Res. Clin. Oncol.* 130:259-265) teach that the frequency of 1G/2G genotypes in gastric cancer patients was similar to those in controls with no differences in degree of tumor invasion, presence of lymph node metastasis and clinical stage; however, the 2G allele may be involved in differentiation of gastric cancer. Further, the Examiner suggests that the A2058 tumor cell lines disclosed in the instant specification are not an appropriate means for examining associations with diseases and that because the mutation and patient are not limited, the specification amounts to an invitation for the skilled artisan to try and follow the disclosed instructions to make and use the claimed invention. Applicants respectfully disagree.

MPEP 2164.01 indicates that the test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation.

As indicated *supra*, Applicants have specifically set forth the Ets transcription factor binding site SNP which results in the increased expression of MMP-1. At the time of filing there was a very high level of appreciation in the art concerning the association between MMP-1 expression and cancer diagnostics and prognostics. For example, it was well-known that the overexpression of MMP-1 was associated with particular cancers in humans and was correlated with a poor prognosis (see, *e.g.*, Murray et al. (1998) *J. Pathol.* 185:256-61 and Murray et al. (1996) *Nat. Med.* 2:461-462; abstracts enclosed herewith). Further, it was established in the art that the expression levels of proteins involved in tumor invasion and metastasis where highly correlative *in vivo* and *in vitro* as evidenced by Stetler-Stevenson et al. ((1990) *J. Biol. Chem.* 265:13933-8; enclosed herewith) which teach that the expression of human tissue inhibitor of metalloproteinases 1 and 2 is the same *in vivo* and in cell culture (*i.e.*, A2058 human melanoma, HT-144 human melanoma, HT-1080 human fibrosarcoma cell lines). Thus, it would be reasonable to one of skill in the art that a SNP which increases the expression of MMP-1 *in vitro* (*e.g.*, the 5'-AAGAT-3' to 5'-AAGGAT-3' mutation) would be useful for diagnosing and prognosticating cancers known to have an elevated level of MMP-1 expression. See page 9, lines 27-31. Therefore, in an effort to facilitate the prosecution of the instant application, Applicants have amended the claims to indicate that the detection of a 5'-AAGAT-3' to 5'-AAGGAT-3' Ets transcription factor binding site SNP in the MMP-1 promoter sequence comprising SEQ ID NO:6 would be useful in the diagnosis and prognosis of human cancer. Support for this amendment can be found throughout the specification and

in particular at the paragraph bridging pages 8 and 9. Accordingly, Applicants believe they have satisfied the enablement requirement for the following reasons:

The breadth of the claims have been narrowed to focus on teachings of the instant specification (*i.e.*, the diagnosis and prognosis of human cancer based on the detection of a 5'-AAGAT-3' to 5'-AAGGAT-3' Ets transcription factor binding site SNP in the MMP-1 promoter sequence comprising SEQ ID NO:6).

While the Examiner suggests several post-filing references that indicate that the 1G/2G mutation may not be indicative of other MMP-1 associated diseases, the teachings of Matsumura et al. post-filing would indicate that the frequency of individuals carrying at least one 2G allele is significantly higher in the diffuse type of gastric cancer than that in the intestinal type (page 263, column 1, para. 2). Further, there was a high level of predictability in the art at the time of filing concerning increased MMP-1 expression levels and the correlation with particular cancers. As the instant invention teaches that increased expression levels are associated with a 5'-AAGAT-3' to 5'-AAGGAT-3' Ets transcription factor binding site SNP in the MMP-1 promoter sequence comprising SEQ ID NO:6 in a melanoma cancer cell, there would be a high level of predictability that this mutation is associated with other cancers known in the art to overexpress MMP-1. MPEP 2164.03 states that "[t]he scope of the required enablement varies inversely with the degree of predictability involved, but even in unpredictable arts, a disclosure of every operable species is not required." Therefore, Applicants are not required to demonstrate that this mutation exists in each and every cancer in every ethnicity and every sex.

The specification provides the necessary guidance (*i.e.*, the 5'-AAGAT-3' to 5'-AAGGAT-3' mutation, a radiolabeled PCR assay, primers, etc.; see Example 5 and the paragraph bridging pages 2 and 3) for one of skill in the art to use the disclosed methods in the diagnosis and prognosis of human cancer.

Further, the specification provides a working example (*i.e.*, the detection of the presence of the 5'-AAGAT-3' to 5'-AAGGAT-3' mutation in A2058 melanoma cells and other tumor cell lines (page 8, lines 18-23)) that correlates with use of the claimed methods of the invention.

With the structure of the Ets transcription factor binding site SNP, as set forth in the amended claims, and the teachings of the instant specification (*e.g.*, primer flanking the claimed mutation), one of skill could readily apply the methods of the present invention to particular cancers known to be associated with the overexpression of MMP-1 without an undue amount of experimentation.

Thus, in view of the claim amendments and accompanying remarks, it is respectfully requested that the rejection of claims based on lack of enablement be reconsidered and withdrawn.

Claims 1-2 have been rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. In particular, the Examiner suggests that it is unclear whether the claims are drawn to a method of diagnosing a MMP-1 disease in a patient or whether the claims are directed to detecting an MMP-1 polymorphism. Further, it is suggested that "the Ets transcription factor binding site single nucleotide polymorphism" lacks proper antecedent basis. To

clarify, Applicants have made the necessary claim amendments. Withdrawal of this rejection is therefore respectfully requested.

V. Rejection of Claims Under 35 U.S.C. §102

Claims 1 and 2 have been rejected under 35 U.S.C. 102(b) as being anticipated by Aho et al. ((1997) *Eur. J. Biochem.* 247:503-510). It is suggested that Aho et al. teach a method of detecting a nucleic acid with an Ets transcription factor binding site polymorphism (*i.e.*, a mutation of the first Ap-1 site).

Because Aho et al. fail to teach or suggest the essential feature of a 5'-AAGAT-3' to 5'-AAGGAT-3' Ets transcription factor binding site SNP in the MMP-1 promoter sequence comprising SEQ ID NO:6, Aho et al. fail to anticipate the instant invention. It is therefore respectfully requested that this rejection be withdrawn.

VI. Conclusion

The Applicants believe that the foregoing comprises a full and complete response to the Office Action of record.

Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

Jane Massey Licata
Registration No. 32,257

Date: **February 15, 2005**

Licata & Tyrrell P.C.
66 E. Main Street
Marlton, New Jersey 08053

(856) 810-1515

1: J Pathol. 1998 Jul;185(3):256-61. Related Articles, Links

InterScience

Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer.

Murray GI, Duncan ME, O'Neil P, McKay JA, Melvin WT, Fothergill JE.

Department of Pathology, University of Aberdeen, U.K. g.i.murray@abdn.ac.uk

The matrix metalloproteinases (MMPs) are a family of closely related proteolytic enzymes which are involved in the degradation of different components of the extracellular matrix. There is increasing evidence to indicate that individual MMPs have an important role in tumour invasion and tumour spread. Monoclonal antibodies specific for MMP-1, MMP-2, or MMP-9 have been produced, using as immunogens peptides selected from the amino acid sequences of individual MMPs. The presence of MMP-1, MMP-2, and MMP-9 in oesophageal cancer was investigated by immunohistochemistry on formalin-fixed, wax-embedded sections of oesophageal cancers. The relationship of individual MMPs to prognosis and survival was determined. MMP-1 was present in 24 per cent of oesophageal cancers, while MMP-2 and MMP-9 were present in 78 and 70 per cent of tumours, respectively. The presence of MMP-1 was associated with a particularly poor prognosis (log rank test 8.46, $P < 0.004$) and was an independent prognostic factor ($P = 0.02$). The identification of individual MMPs in oesophageal cancer provides a rational basis for use in the treatment of oesophageal cancer of MMP inhibitors which are currently undergoing clinical trial.

PMID: 9771478 [PubMed - indexed for MEDLINE]

Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer.

Murray GI, Duncan ME, O'Neil P, Melvin WT, Fothergill JE.

Department of Pathology, University of Aberdeen, Aberdeen, UK.

Colorectal cancer is one of the commonest malignant tumors and has a relatively poor prognosis. The outcome depends on the extent of local and particularly metastatic tumor spread. The matrix metalloproteinases (MMPs) are a family of closely related enzymes that degrade the extracellular matrix and are considered to be important in facilitating tumor invasion and spread (1-3). Using immunohistochemistry we have investigated the occurrence in colorectal cancer of MMP-1 (interstitial collagenase). Our monoclonal antibody was prepared against a synthetic peptide corresponding to an amino acid sequence specific for MMP-1 and was selected to react in formalin-fixed wax-embedded sections, thus allowing use in diagnostic histopathology and also enabling access to archival material. We found that the presence of MMP-1 in colorectal cancer is associated with a poor prognosis (P = 0.006) and has prognostic value independent of Dukes stage. One MMP inhibitor that strongly inhibits MMP-1 has already been shown to inhibit growth of human colon cancer xenografts in nude mice (4). Our results suggest that treatment of those individuals whose colon tumors produce MMP-1 with MMP inhibitors is a therapeutic strategy worth pursuing.

PMID: 8597958 [PubMed - indexed for MEDLINE]

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 23, Issue of August 15, pp. 13933-13938, 1990
Printed in U.S.A.

Tissue Inhibitor of Metalloproteinases-2 (TIMP-2) mRNA Expression in Tumor Cell Lines and Human Tumor Tissues*

(Received for publication, March 14, 1990)

William G. Stetler-Stevenson‡, Peter D. Brown, Maurizio Onisto, Anna T. Levy, and Lance A. Liotta

From the Tumor Invasion and Metastasis Section, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20892

Human tissue inhibitor of metalloproteinase-2 (TIMP-2) was cloned and sequenced from an A2058 human melanoma cell cDNA library. When the sequence was compared with that of human TIMP-1 at both the nucleotide and deduced amino acid levels, the homology appeared closer at the protein level than at the nucleotide level, suggesting that these inhibitors diverged early in the evolution of this gene family. Comparison of the deduced amino acid sequence for TIMP-2 with that of human TIMP-1 shows that there are two regions in which the similarity is below the overall average of 66%. It is postulated that these regions are responsible for the unique ability of TIMP-2 to bind to the latent form of the 72-kDa type IV collagenase. Polyclonal anti-TIMP-2 antisera recognized TIMP-2 but not TIMP-1 on immunoblotting.

Northern blot analysis of RNA from A2058 human melanoma, HT-144 human melanoma, HT-1080 human fibrosarcoma, and WI-38 fetal lung fibroblast cell lines demonstrated two distinct transcripts of 1.0 and 3.5 kilobases (kb) for *timp-2* mRNA. Both transcripts are down-regulated in response to transforming growth factor-β but are unchanged in response to phorbol ester treatment. This is in contrast to the up-regulation of *timp-1* transcripts by these agents and indicates that *timp-2* and *timp-1* are independently regulated in cell culture. Northern blot analyses of matched normal and tumor tissue samples from five cases of human colorectal carcinoma were performed. Normal and tumor tissues contain both the 1.0- and 3.5-kb transcripts. However, in the tissue samples the ratio of the 3.5-kb transcript to the 1.0-kb transcript was markedly elevated. No evidence of down-regulation of *timp-2* transcript levels was noted in the tumor tissues. This is in contrast to the elevated *timp-1* transcript levels seen in these tumor samples. Thus, *timp-2* mRNA transcript levels are differentially regulated from *timp-1* levels *in vivo* as well as in cell culture.

The collagenase family enzymes are a group of metalloproteinases which are secreted in the zymogen form and degrade both the collagenous and noncollagenous components of the extracellular matrix. The overproduction and unrestrained activity of these enzymes have been linked to a variety of pathologic conditions such as rheumatoid arthritis and malignant conversion of tumor cells (Okada *et al.*, 1986; Harris *et al.*, 1984; Werb *et al.*, 1977; Liotta *et al.*, 1980; Kalebic *et al.*, 1983). The down-regulation of metalloproteinase collagenolysis and proteolysis may occur through naturally occurring inhibitor proteins, such as the tissue inhibitors of metalloproteinases (TIMPs).[1]

TIMP-1 is a glycoprotein with an apparent molecular size of 28.5 kDa which forms a complex of 1:1 stoichiometry with activated interstitial collagenase, activated stromelysin, and the 92-kDa type IV collagenase (Welgus and Stricklin, 1983: Welgus *et al.*, 1985a; Wilhelm *et al.*, 1989). The gene coding for TIMP-1 has been cloned, sequenced, and mapped to the X chromosome (Carmichael *et al.*, 1986; Docherty *et al.*, 1985; Mullins *et al.*, 1988; Mahtani and Willard, 1988). The secreted protein has 184 amino acids and six intramolecular disulfide bonds. The same cells which produce interstitial collagenase are capable of synthesizing and secreting TIMP-1 (Welgus *et al.*, 1985b; Herron *et al.*, 1986). Thus, the net collagenolytic activity for these cell types is the result of the balance between activated enzyme levels and TIMP-1 levels. Studies have shown an inverse correlation between TIMP-1 levels and the invasive potential of murine and human tumor cells (Khokha *et al.*, 1989).

Recently we have isolated, purified, and determined the complete primary structure of a second member of the TIMP family, TIMP-2 (Stetler-Stevenson *et al.*, 1989). TIMP-2 is a 21-kDa protein which selectively forms a complex with the latent proenzyme form of the 72-kDa type IV collagenase (Stetler-Stevenson *et al.*, 1989; Goldberg *et al.*, 1989). The secreted protein has 192 amino acid residues and is not glycosylated. TIMP-2 shows an overall 71% similarity to TIMP-1 at the amino acid sequence level. The positions of the 12 cysteine residues are conserved with respect to those present in TIMP-1, as are three of the four tryptophan residues. TIMP-2 inhibits the type IV collagenolytic activity and the gelatinolytic activity associated with the 72-kDa enzyme (Stetler-Stevenson *et al.*, 1989). Inhibition studies demonstrated that complete enzyme inhibition occurred at a 1:1 molar ratio of TIMP-2 to activated 72-kDa type IV collagenase (Stetler-Stevenson *et al.*, 1989). Thus, unlike TIMP-1, TIMP-2 is capable of binding to both the latent and activated forms of type IV collagenase. Cell culture studies using cell lines that produce a variety of collagenase family enzymes, as well as both TIMP-1 and TIMP-2 suggest that TIMP-2 preferentially interacts with the 72-kDa type IV collagenase (Stetler-Stevenson *et al.*, 1989; Goldberg *et al.*,

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05593.

‡ To whom correspondence should be addressed: Laboratory of Pathology, NCI, Bldg. 10, Rm. 2A33, 9000 Rockville Pike, Bethesda, MD 20892. Tel.: 301-496-2687.

[1] The abbreviations used are: TIMP, tissue inhibitor of metalloproteinase; TGF-β1, transforming growth factor β1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HPLC, high performance liquid chromatography; kb, kilobase(s).

1989). Thus, like interstitial collagenase activity which is the balance of activated enzyme and TIMP-1, the net 72-kDa type IV collagenase activity may depend upon the balance between the levels of activated enzyme and TIMP-2.

Augmented type IV collagenolytic activity has been associated with the metastatic phenotype in a number of experimental systems. This could possibly be due to the increased production and activation of the 72-kDa type IV collagenase enzyme. However, decreased production of TIMP-2 could also result in greater effective enzyme activity. To examine the regulation of TIMP-2 we have isolated and sequenced a cDNA clone for human TIMP-2. Comparison of the cDNA sequence of *timp-2* with that of *timp-1* suggests that these inhibitors diverged early in the evolution of the TIMP family. We have used this probe to measure the levels of *timp-2* mRNA in human tumor cell lines and the effects of phorbol ester and transforming growth factor β (TGF-β1) treatment on *timp-2* mRNA levels. These effects were contrasted with those noted for *timp-1* mRNA levels. Finally, we have examined *timp-2* and *timp-1* mRNA levels in a series of human colon adenocarcinomas and adjacent normal colonic mucosa.

EXPERIMENTAL PROCEDURES

Human Melanoma Cell cDNA Library Preparation, Screening, and DNA Sequencing—Oligo(dT)-select poly(dA) mRNA was prepared from human A2058 melanoma cells using standard methods. 1 μg of purified mRNA was used to prepare double-stranded cDNA using a commercially available cDNA synthesis kit (Amersham Corp.). This cDNA was methylated using *Eco*RI methylase (Promega), linked to *Eco*RI linkers (Promega), restricted with *Eco*RI, and ligated to *Eco*RI-digested λ-GEM-4 (Promega). The ligations were packaged (Gigapack Gold, Strategene) and the optimal reactions were pooled to give 1.5 $\times$ 10^6 recombinants. 7.5 $\times$ 10^5 recombinants were screened using oligonucleotide 27–40. Oligonucleotide 27–40, a 45-mer, with the sequence, 5'GAGAAGGAGGTGGACTCTGGCAATGACATCTATGGCAACAACATC3', corresponds to the reverse translation of residues 27 through 40 of the previously sequenced TIMP-2 protein. Oligonucleotide 27–40 was synthesized on a Biosearch 8700 DNA synthesizer by means of β-cyanoethyl phosphoramidite chemistry and was labeled using γ-[^{32}P]ATP (Amersham Corp.) and T4 kinase (Bethesda Research Labaoratories). Library screening was performed using standard techniques. DNA sequencing was performed using dideoxy methodology and [^{35}S]dATP (Du Pont-New England Nuclear).

Cell Culture, RNA Isolation, and Northern Blot Analysis—All cell lines except A2058 melanoma cells were obtained from the American Type Culture Collection, Rockville, MD. HT-1080 human fibrosarcoma cells, Wi-38 human embryonic lung fibroblasts, HT-144 human melanoma, and A2058 human melanoma cells were grown to 80% confluence in Dulbecco's modified Eagle's medium (GIBCO). The medium was then replaced with Dulbecco's modified Eagle's medium supplemented with 0.5% ITS$^+$ (Collaborative Research) and 25 μg/ml gentamycin. The medium was changed after 4 h and culture continued for 20 h prior to the addition of 10 ng/ml 12-*O*- tetradecanoylphorbol-13-acetate (TPA, Sigma) or 5 ng/ml TGF-β1 (R & D Systems).

Total cytoplasmic RNA was isolated from cell lines as described by Gough (1988). mRNA was isolated using the FAST-TRACK mRNA isolation kit (Invitrogen). Tissue mRNA was isolated from frozen tissue fragments. Tissue fragments were obtained from partial colectomy specimens at the time of surgery. The pathologic diagnosis of all five cases was invasive adenocarcinoma. Tissue samples were also obtained from adjacent uninvolved mucosa. Frozen tissue was pulverized in liquid N_2 using a mortar and pestle. The tissue powder was then dissolved in 4 M guanidine isothiocyanate, 3 M sodium acetate, 0.84% β-mercaptoethanol, pH 6.0. Total cytoplasmic RNA was isolated by pelleting through 5.7 M cesium chloride, 3 M sodium acetate, pH 6.0. Aliquots of RNA were applied to formaldehyde, 1% w/v agarose gels and electrophoresed before transfer onto Nytran filters (Schleicher & Schuell). The RNA was UV cross-linked to the filter and hybridized using standard conditions. The cDNA probes were labelled with α-[^{32}P]dCTP using a random primer labeling kit (Bethesda Research Laboratories). Filters were autoradiographed at −80 °C, and quantitation on developed film was performed by scanning densitometry using an LKB 2202 laser densitometer.

A TIMP-1 cDNA probe was prepared by polymerase chain amplification reaction using cDNA prepared from oligo(dT)-selected mRNA from HT-1080 TPA-treated cells. The cDNA was prepared using a cDNA synthesis kit (Bethesda Research Laboratories) according to the manufacturer's directions. The primers for this amplification were also synthesized on the Biosearch 8700 DNA synthesizer and had the following sequences corresponding to sequences reported in the human TIMP-1 cDNA clone (Carmichael *et al.*, 1986): primer 82 (bases 118–146), 5'TGCACCTGTGTCCCACCCCACCCACAGACG3'; primer 83 (complement to bases 640–669), 5'GGCTATCTGGGACCGCAGGGACTGCCAGGT3'. The polymerase chain amplification reaction was performed for 40 cycles using 55 °C annealing temperature and standard conditions for denaturation and extension. This reaction consistently yielded a single 551-base pair product (P551) encoding for the mature TIMP-1 protein sequence.

TIMP-2 Purification and Antibody Preparation—TIMP-2 was purified from human melanoma A2058 cell-conditioned media by gelatin affinity chromatography and reverse-phase HPLC as previously described (Stetler-Stevenson *et al.*, 1989). The purified human TIMP-2 was used as the antigen for preparation of polyclonal rabbit anti-human TIMP-2 antibodies using standard immunization protocols previously described (Hoyhtya *et al.*, 1988).

RESULTS

We have previously determined the primary structure of the TIMP-2 protein by direct amino acid sequencing (Stetler-Stevenson *et al.*, 1989). This information was used to prepare a synthetic oligonucleotide probe, oligo 27–40, which was used to screen a cDNA library constructed from mRNA isolated from human A2058 melanoma cells. Ten clones were isolated, and the nucleotide sequence of the cDNA insert in the longest clone, pT2-MO1, is presented in Fig. 1. The insert contains 1062 base pairs excluding the poly(A) tail and encodes for the pro-TIMP-2 protein of 220 amino acids. This protein se-

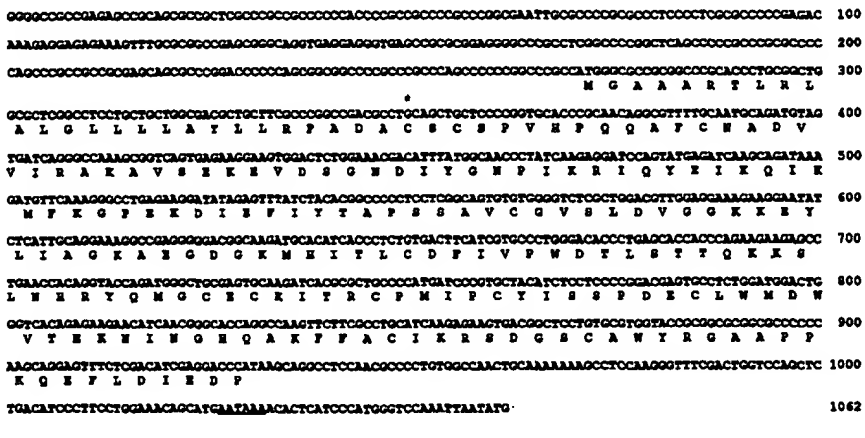

FIG. 1. **Nucleotide sequence of human TIMP-2 cDNA.** The cDNA insert of clone pT2-M01 was sequenced in both directions using dideoxy methodology. The predicted amino acid sequence is shown under the DNA sequence. The putative polyadenylation signal is *underlined*.

quence includes a 26-residue signal peptide sequence and a mature TIMP-2 protein of 194 amino acids. The 130-nucleotide-long 3′-untranslated region contains a polyadenylation signal 30 bases upstream from the 3′ end of the RNA.

Comparison of the amino acid sequence of TIMP-2 deduced from the cDNA clone with that determined by direct amino acid sequencing of overlapping endoproteinase-derived peptide fragments shows excellent agreement. The original sequence contained only 192 amino acids. The previously unidentified residues correspond to the glycyl residue at position 92 and the prolyl residue at the carboxyl terminus. Other changes are noted in Fig. 2. The homology of mature TIMP-2 with TIMP-1 at the predicted protein level is 37.6% identity and 65.6% overall similarity.

Rabbit anti-human TIMP-2 polyclonal antibodies were used in an immunoblot comparison of bovine TIMP-1 and human TIMP-2. As shown in Fig. 3, bovine TIMP-1 migrates at approximately 28 kDa, as previously reported for this glycosylated protein, compared with TIMP-2 which migrates at 21 kDa and is unglycosylated. Rabbit polyclonal anti-TIMP-2 antibodies failed to detect TIMP-1 but showed excellent reactivity with TIMP-2.

Northern blot analysis of oilgo(dT)-selected mRNA isolated from the A2058 human melanoma cell line revealed two specific *timp-2* mRNA species with approximate sizes of 3.5 and 1.0 kb (Fig. 4). These *timp-2* transcripts are clearly distinguished from the 0.9-kb *timp-1* transcript detected with the P551 probe. No evidence of cross-hybridization between the *timps* was observed under the hybridization and stringent wash conditions utilized. Both *timp-2* mRNA species were also detected in RNA isolated from human Wi-38 fibroblasts, HT-1080 fibrosarcoma, and HT-144 melanoma cells. The 3.5-kb *timp-2* transcript predominated in the fibroblast and fibrosarcoma cell lines with only low levels of the 1.0-kb species detectable (Fig. 5).

Treatment of cells with TPA (10 ng/ml) for 48 h failed to significantly modulate either *timp-2* transcript level in the melanoma cell lines tested (Fig. 5). Both *timp-2* transcripts showed a 2-fold induction by TPA in the normal fibroblast cell line Wi-38 and a 2-fold reduction in the fibrosarcoma cell line HT-1080. In contrast *timp-1* transcript levels were increased 2-fold by TPA treatment in both melanoma cell lines as well as in the HT-1080 fibrosarcoma cells. Phorbol ester treatment of the Wi-38 fibroblast cell line resulted in an 8-

```
CSCSPVHPQQ APCNADVVIR AKAVSEKEVD SGNDIYGNPI KRIQYEIKQI  CSC 21K
CSCSPVHPQQ APCNADVVIR AKAVSEKEVD SGNDIYGNPI KRIQYEIKQI  TIMP 2

KKFKGIEKDI EFIYTAPSSA VCGVELDVGG KKEYLIAGKA E DGKRHITL  CSC 21K
KMFKGPEKDI EFIYTAPSSA VCGVSLDVGG KKEYLIAGKA EGDGKMHITL  TIMP 2

CDFIVPWDTL STTQKKSLNH RYQQGCECKI TRCPMIPCYI SSPDECLWTD  CSC 21K
CDFIVPWDTL STTQKKSLNH RYQMGCECKI TRCPMIPCYI SSPDECLMMD  TIMP 2

WVTEKNINGH QAKFFACIKR SDGSCAWYRG AAPPKQEFLD IED         CSC 21K
WVTEKNINGH QAKFFACIKR SDGSCAWYRG AAPPKQEFLD IEDP        TIMP 2
```

FIG. 2. **Comparison of TIMP-2 deduced amino acid sequence and CSC-21K (Stetler-Stevenson *et al.*, 1989).** CSC-21K primary structure was determined directly using a Porton Instruments 2020 gas-phase protein sequenator and phenylhydantoin derivative identification on a Beckman System Gold HPLC unit equipped with a 0.46 × 25-cm Beckman ODS column. Comparison shows 96% identity of these sequences. *Asterisks* identify changes in the TIMP-2 sequence.

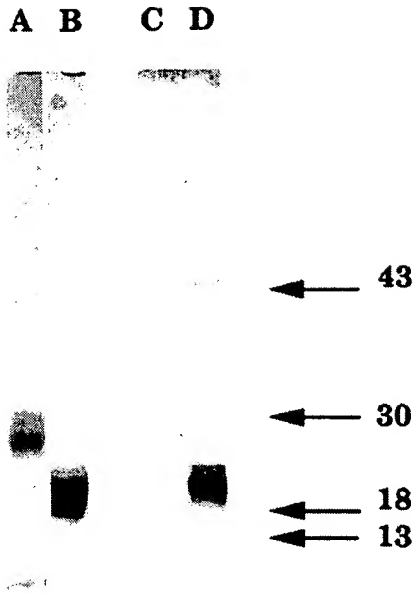

FIG. 3. **Immunoblot analysis of TIMP-1 and TIMP-2 using anti-TIMP-2 polyclonal antibodies.** *Lanes A* and *C* contained 1 μg of TIMP-1. *Lanes B* and *D* contained 1 μg of TIMP-2. *Lanes A* and *B* were silver-stained following electrophoresis. *Lanes C* and *D* were stained with rabbit anti-human TIMP-2 antisera (1:200) followed by goat anti-rabbit horseradish peroxidase complex after transfer to Immobilon membrane (Millipore).

FIG. 4. **Northern blot analysis of TIMP-2 mRNA expression in cultured cell lines.** Oligo(dT)-selected RNA was isolated from cells as described under "Experimental Procedures." After transfer to Nytran filters RNA was hybridized with ^{32}P-labeled probe pT2-MO1, specific for *timp-2*, or alternatively, probe P551, specific for *timp-1*. Relative positions of the 28 and 18 S ribosomal RNA bands are noted as are the approximate transcript sizes.

fold induction of the *timp-1* transcript levels. These findings are consistent with the induction of *timp-1* by this agent as previously reported (Edwards *et al.*, 1985; Murphy *et al.*, 1985; Welgus *et al.*, 1985b).

Treatment of cells with TGF-β1 for 48 h resulted in a clearly detectable decrease in *timp-2* mRNA levels in all cell lines tested except the Wi-38 fibroblasts (Fig. 5). The 3.5- and 1.0-kb *timp-2* transcripts showed equal TGF-β1-induced decreases in steady-state levels, and there was no indication

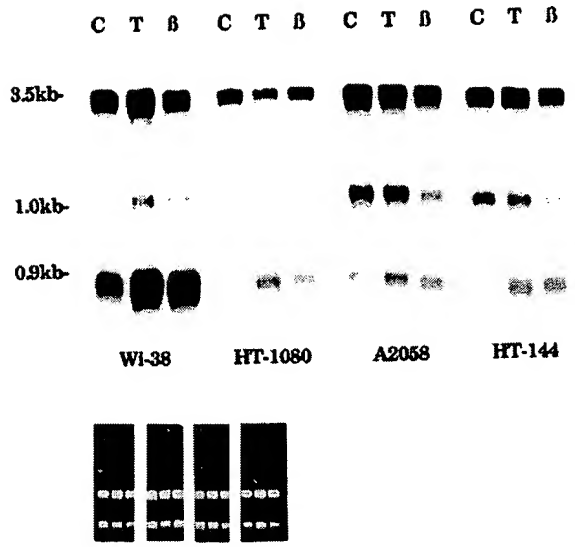

FIG. 5. **Northern blot analysis of total cytoplasmic RNA isolated from Wi-38 fibroblasts, HT-1080 fibrosarcoma, HT-144 melanoma, and A2058 melanoma untreated cells (*lanes C*) or following 48-h treatment of the cells with either TPA (10 ng/ml, *lanes T*) or TGF-β1 (5 ng/ml, *lanes β*).** Equal amounts of RNA (5 μg) were loaded, and the ethidium bromide-stained gel is shown as a control (*inset*). Autoradiographs of several different exposures were scanned using a laser densitometer for quantitation.

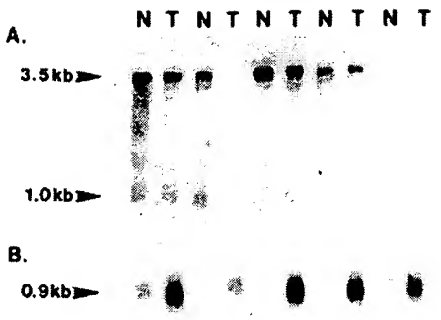

FIG. 6. **Northern blot analysis of five human colorectal tumors and adjacent normal mucosa.** RNA (5 μg) of each sample was electrophoresed and transferred as described under "Experimental Procedures." *Lanes T* contain RNA from the invasive colorectal tumors. *Lanes N* contain RNA from the corresponding adjacent normal mucosa.

of differential repression by TGF-β1. Treatment of A2058 melanoma cells with TGF-β1 reduced steady-state *timp-2* transcript levels to 46 and 59% of control values for the 1.0 kb and 3.5-kb transcripts, respectively. Treatment of HT-144 cells with TGF-β1 resulted in reduction of *timp-2* transcripts to 42 and 47% of control levels for the 1.0 and 3.5-kb message, respectively. Both *timp-2* transcripts showed a more moderate TGF-β1-induced reduction in the HT-1080 fibrosarcoma cell line of 25% from control levels. These effects are in contradistinction to the results observed for *timp-1* message levels. In the tumor cell lines (HT-144, HT-1080, and A2058) TGF-β1 induced an increase in *timp-1* steady-state transcript levels to 150% of control values. Treatment of Wi-38 fibroblasts with TGF-β1 resulted in a 6-fold increase of message levels. These effects for *timp-1* are consistent with the report by Overall *et al.*, (1989) which demonstrated that TGF-β induces *timp-1* mRNA levels. These observations clearly demonstrate that *timp-2* and *timp-1* are differentially regulated in all four cell lines tested.

Northern blot analyses of tissue from five primary human colorectal tumors and patient-matched adjacent normal mucosa were performed using both the *timp-1* and *timp-2* probes (Fig. 6). Ethidium bromide staining of the formaldehyde gels prior to transfer demonstrated equal loading of RNA in all lanes (data not shown). The results demonstrate that both the 1.0- and 3.5-kb *timp-2* transcripts are present but that the 1.0-kb message level is markedly reduced compared with that seen in the RNA isolated from cultured human tumor cells. Transcript levels for *timp-2* show little correlation with tissue origin (normal *versus* tumor). In most tumor samples the *timp-2* transcript levels show no difference from the adjacent normal tissue, with the exception of tumor T2 in which there is a marked decrease in *timp-2* message levels compared with that seen in the adjacent normal colonic mucosa N2. These data suggest that primary colon adenocarcinomas may be heterogeneous with respect to the levels of *timp-2*. However, *timp-1* transcript levels are obviously elevated in all tumor tissue samples, including sample T2, compared with normal adjacent mucosa. Again, these observations suggest that *timp-2* and *timp-1* are independently regulated.

DISCUSSION

Isolation and sequencing of a cDNA clone for human TIMP-2 was performed. Characterization of the TIMP-2 cDNA clone pT2-MO1 confirmed that TIMP-2 is a unique gene product independent of TIMP-1. The protein sequence predicted from the TIMP-2 cDNA is in excellent agreement (95%) with the amino acid sequence obtained for this protein by direct protein sequencing (Stetler-Stevenson *et al.*, 1989). Pustell Matrix analysis of the homology distribution between these two predicted protein sequences using a cut-off value of 66% and an 8-amino acid overlap demonstrates that there are two areas in which the homology falls below this average value (Fig. 7*A*). TIMP-2 shows a distinct preference for binding to the latent form of the 72-kDa type IV collagenase in the presence of both other latent metalloproteinases and TIMP-1 (Stetler-Stevenson *et al.*, 1989; Goldberg *et al.*, 1989). However, both forms of TIMP will inhibit activated type IV collagenase. Thus regions of amino acid sequence that are highly conserved between these proteins, such as those that exceed the overall homology value of 66%, may be responsible for the known shared functions of these proteins, inhibition of the activated collagenase family of enzymes. Areas of low homology are likely to be responsible for those functions which are unique for individual TIMP molecules. Thus, the regions of low homology between residues 45 and 70 and the carboxyl terminus of TIMP-2 may be responsible for the binding of TIMP-2 to the latent form of the 72-kDa type IV collagenase. Such regions must exist and account for the failure of TIMP-1 antibodies to detect TIMP-2, as previously reported (Goldberg *et al.*, 1989), as well as the failure of TIMP-2 antibodies to detect TIMP-1 as demonstrated in the current report (Fig. 3).

Comparison of the cDNA sequences of human *timp-2* with human *timp-1* shows little homology considering that seen at the amino acid level (Fig. 7*B*). This result implies that these genes diverged early in the evolution of this gene family. The lack of homology at the cDNA level may also explain why *timp-2* mRNA transcripts are not detected in Northern blot analyses using *timp-1* probes and also why screening cDNA libraries with *timp-1* probes fails to yield *timp-2* clones.

Northern blot analyses of oligo(dT)-selected poly(A) RNA as well as total cytoplasmic RNA from human tumor cell lines detect the presence of two transcripts when probed with the full-length *timp-2* cDNA clone pT2-MO1. The origin of these

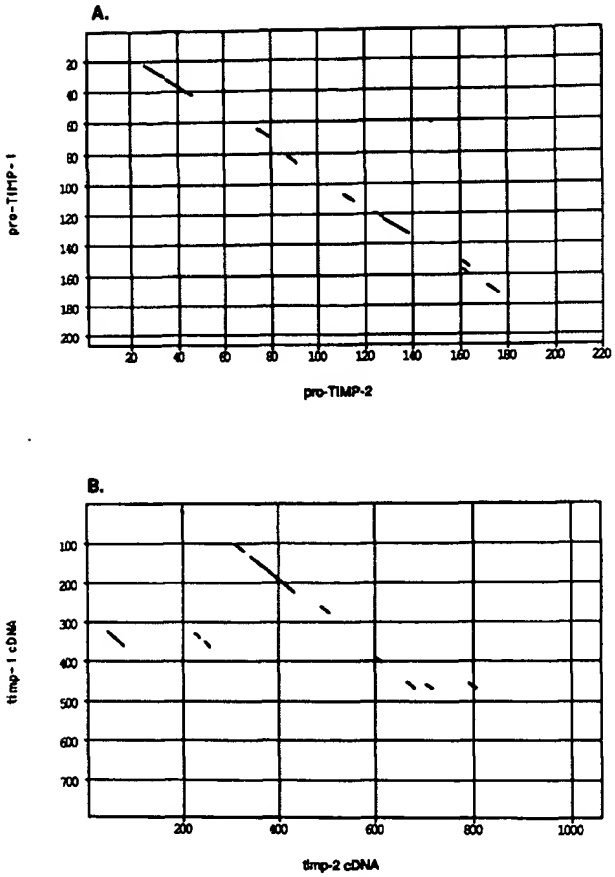

FIG. 7. **Homology comparison of TIMP-2 and TIMP-1 at the amino acid (*A*) and nucleotide levels (*B*).** *A*, deduced amino acid sequences of TIMP-2 and TIMP-1 were compared using a Pustell Scoring Matrix. The analysis was performed using a cut-off value of 66% homology and an 8-amino acid overlap. The *line* denotes regions in which the homology exceeds the average value of 66% homology between these two proteins. *B*, comparison of the nucleotide sequences of *timp-2* and *timp-1*. Analysis was performed using a Pustell Scoring Matrix, with a hash value of 4 and a window of 30. The *line* indicates regions of identity. The analysis if performed for *timp-1 versus timp-1* or *timp-2 versus timp-2* gives a *solid line* on the diagonal, indicating complete identity. This demonstrates that *timp-2* is a unique gene product distinct from *timp-1*.

two specific transcripts remains to be determined. The size difference is too large to be easily accounted for by differences in 3′-polyadenylation, although large differences in 3′-portions of the pro-α2(I) collagen transcripts, attributable to alternative polyadenylation signals, have been observed (Myers *et al.*, 1983). It is possible that alternative 5′-untranslated regions could account for the different transcript sizes, as has been demonstrated for insulin-like growth factor II mRNAs (Irminger *et al.*, 1987).

TGF-β1 has been demonstrated to increase *timp-1* mRNA levels in human gingival fibroblasts (Overall *et al.*, 1989). In the presence of other growth factors, TGF-β also has a selective reciprocal effect on interstitial collagenase and *timp-1* expression (Edwards *et al.*, 1987). TGF-β selectively represses the induction of interstitial collagenase but interacts synergistically to superinduce TIMP-1. The results of the present report are consistent with these previous observations. Both TPA and TGF-β1 induced increases in *timp-1* transcript levels over basal levels. This suggests that *timp-1* up-regulation occurs in a similar fashion in the melanoma and fibrosarcoma cells studied in the present report as in the fibroblast cells studied by other investigators. We have previously demonstrated that TGFβ-1 induces the 72-kDa type IV collagenase mRNA and protein levels in the same tumor cell lines studied in the present report.[2] We now demonstrate that TGF-β1 decreases *timp-2* mRNA transcript levels. Thus, TGF-β1 treatment has an opposite effect on *timp-2* compared with the 72-kDa type IV collagenase transcript levels in human tumor cells. This suggests that TGF-β1 treatment may result in augmented type IV collagenolytic activity due to up-regulation of the enzyme coupled with down-regulation of an associated inhibitor, TIMP-2. This could result in an enhanced invasive phenotype of tumor cells treated with TGF-β1, although TGF-β1 does induce an increase in *timp-1* transcript levels. These observations demonstrate the complex multilevel regulation of type IV collagenolytic activity. However, it is clearly evident that the transcriptional regulation of *timp-2* is independent of *timp-1*.

Northern blot analyses of human colorectal tumor and adjacent normal tissues again demonstrated two mRNA transcripts when probed with the *timp-2* cDNA clone. However, in the RNA from these tissue samples the 3.5-kb transcript clearly predominates, with only trace amounts of the 1.0-kb message detectable in the normal tissues. There was no correlation of *timp-2* transcript levels and adenocarcinoma tissues. However, transcript levels for *timp-1* show a distinct correlation with the malignant tumor samples. All adenocarcinoma tissue samples showed elevated *timp-1* levels compared with adjacent normal colonic mucosa. This result was highly unexpected in light of the malignant nature of the tumor tissues examined and the report of Khokha *et al.* (1989) which demonstrated an inverse correlation between *timp-1* mRNA levels and the invasive phenotype. Immunohistochemical studies of the distribution of TIMP-1 protein in these tumor samples will be helpful in developing an understanding of these observations.

In summary, we have cloned and sequenced a full-length cDNA which encodes the pro-TIMP-2 protein. At the protein level there is a marked homology between members of the TIMP family. However, at the nucleotide level this homology is notably less, suggesting early divergence of these genes. Finally, the data presented clearly demonstrate that *timp-2* is regulated independently of *timp-1*, both in cell culture as evidenced by studies using TPA and TGF-β1 and *in vivo* as evidenced by comparison of transcript levels for these inhibitors in human colon adenocarcinoma tissue and adjacent normal colonic mucosa.

Acknowledgments—We wish to thank M. Sveda and G. Zewe of Biotechnology Research Institute/Organon Teknika for their technical help with the isolation and sequencing of the cDNA clones. We thank M. Sobel and V. Cioce for the RNA samples from the colorectal tumors, and E. Unsworth for the oligonoucleotide synthesis.

Note Added in Proof—The cDNA sequence reported is identical to the human metalloproteinase inhibitor (MI) sequence reported by Boone *et al.* (Boone, T. C., Johnson, M. J., DeClerck, Y. A., and Langley, K. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2800–2804.)

REFERENCES

Carmichael, D. F., Sommer, A., Thompson, R. C., Anderson, D. C., Smith, C. G., Welgus, H. G., and Stricklin, G. P. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 2407–2411

Docherty, A. J. P., Lyons, A., Smith, B. J., Wright, E. M., Stephens, P. E., Harris, T. J. R., Murphy, G., and Reynolds, J. J. (1985) *Nature* **318**, 66–69

[2] P. D. Brown, A. T. Levy, I. M. K. Marguiles, L. A. Liotta, and W. G. Stetler-Stevenson, *Cancer Res.*, submitted for publication.

Edwards, D. R., Parfett, C. L. J., and Denhardt, D. T. (1985) *Mol. Cell. Biol.* **5**, 3280–3288

Edwards, D. R., Murphy, G., Reynolds, J. J., Whitham, S. E., Docherty, A. J. P., Angel, P., and Heath, J. K. (1987) *EMBO J.* **6**, 1899–1904

Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S., and He, C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 8207–8211

Gough, N. M. (1988) *Anal. Biochem.* **173**, 93–95

Harris, E. D., Welgus, H. G., and Krane, S. M. (1984) *Collagen Relat. Res.* **4**, 493–512

Herron, G. S., Banda, M. J., Clark, E. J., Gavrilovic, J., and Werb, Z. (1986) *J. Biol. Chem.* **261**, 2814–2818

Hoyhtya, M., Turpeenniemi-Hujanen, T., Stetler-Stevenson, W. G., Krutzsch, H., Tryggvason, K., and Liotta, L. A. (1988) *FEBS Lett.* **233**, 109–113

Irminger, J.-C., Rosen, K. M., Humbel, R. E., and Villa-Komaroff, L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6330–6334

Kalebic, T., Garbisa, S., Glaser, B., and Liotta, L. A. (1983) *Science* **221**, 281–283

Khokha, R., Waterhouse, P., Yagel, S., Lala, P. K., Overall, C. M., Norton, G., and Denhardt, D. T. (1989) *Science* **243**, 947–950

Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Schaffie, S. (1980) *Nature* **284**, 64–68

Mahtani, M. M., and Willard, H. F. (1988) *Genomics* **2**, 294–301

Mullins, L. J., Grant, S. G., Stephenson, D. A., and Chapman, V. M. (1988) *Genomics* **3**, 187–194

Murphy, G., Reynolds, J. J., and Werb, Z. (1985) *J. Biol. Chem.* **260**, 3079–3083

Myers, J. C., Dickson, L. A., de Wet, W. J., Bernard, M. P., Chu, M.-L., Di Liberto, M., Pepe, G., Sangiogi, F. O., and Ramirez, F. (1983) *J. Biol. Chem.* **258**, 10128–10135

Okada, Y., Nagase, H., and Harris, E. D. (1986) *J. Biol. Chem.* **261**, 14245–14255

Overall, C. M., Wrana, J. L., and Sodek, J. (1989) *J. Biol. Chem.* **264**, 1860–1869

Stetler-Stevenson, W. G., Krutzsch, H. C., and Liotta, L. A. (1989) *J. Biol. Chem.* **264**, 17374–17378

Welgus, H. G., and Stricklin, G. P. (1983) *J. Biol. Chem.* **253**, 12259–12264

Welgus, H. G., Jeffery, J. J., Eisen, A. Z., Roswit, W. T., and Stricklin, G. P. (1985a) *Collagen Relat. Res.* **5**, 167–179

Welgus, H. G., Campbell, E. J., Bar-Shavit, Z., Senior, R. M., and Teitelbaum, S. L. (1985b) *J. Clin. Invest.* **76**, 219–224

Werb, Z., Mainardi, C. L., Vater, C. A., and Harris, E. D. (1977) *N. Engl. J. Med.* **296**, 1017–1023

Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., and Goldberg, G. I. (1989) *J. Biol. Chem.* **264**, 17213–17221